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Proteins of Morula-like Cells in Hemolymph of the Giant Clam, *Tridacna derasa*

KOJI NAKAYAMA*, MASAHARU ISHIKURA, AND TADASHI MARUYAMA*

Marine Biotechnology Institute, Shimizu Laboratories, 1900 Sodeshi, Shimizu, Shizuoka 424, Japan

Abstract. The morula-like cell, a hemocyte packed with many large (about 3 μm in diameter) electron-dense granules, is found only in the hemolymph of giant clams belonging to the Tridacnidae. To clarify the function of the morula-like cell, we investigated its proteins, especially those found in the large granules. Proteins with molecular weights of 64 kDa, 17 kDa and 7.4 kDa were found to be specific to this type of hemocyte. N-terminal amino acid sequence analysis revealed that the 17-kDa and 7.4-kDa proteins were novel proteins rich in aromatic amino acids. Rabbit polyclonal antibody against a synthetic peptide of the 7.4-kDa protein reacted not only with that protein but also with a larger molecular weight (about 16-kDa) protein in the morula-like cell. Examination of the N-terminal amino acid sequences showed that the 16-kDa protein is distinct from the 17-kDa protein, and Western blot analysis suggested that it is a precursor of the 7.4-kDa protein. The zooxanthellate portion of clam mantle and kidney contained proteins immunoreactive to the antibody, but the azooxanthellate portion of the mantle did not contain any immunoreactive protein. These results suggest that the morula-like cells interact with the zooxanthellae.

Introduction

Giant clams, bivalve molluscs in the family Tridacnidae, harbor symbiotic zooxanthellae in the zooxanthellal tube, which arises from the stomach (Norton *et al.*, 1992). Carbon photosynthetically fixed by the algae is reportedly translocated to the animal (Goreau *et al.*, 1973). Why the zooxanthellae are not rejected by the host clam is an open question. To understand the mechanisms underlying the stable association between the host and symbiont, it is important to study the self-nonself recognition system and the self-defense system of the host. In animals, blood cells, or hemocytes, participate in these systems.

Several types of hematocyte have been identified in the hemolymph of tridacnid species: two types in *Tridacna maxima* (Reade and Reade, 1976) and, more recently, three types in *T. crocea* (Nakayama *et al.*, 1997). The eosinophilic granular hemocyte in *T. crocea,* which corresponds to the type I cell in *T. maxima,* has phagocytic ability against foreign materials. The agranular cell in *T. crocea* seems to correspond to the bivalve hyaline cell reviewed by Cheng (1981, 1984). This type of hemocyte adheres to glass surfaces and contains electron-lucent granules and only a few electron-dense granules. The agranular cells were mainly found in the core space of the clots formed by the aggregation of hemocytes after exposure to seawater. (Nakayama *et al.*, 1997). The morula-like cell in *T. crocea,* which corresponds to the type II cell in *T. maxima,* is packed with many large (about 3 μm in diameter), electron-dense granules. Hemocytes packed with large granules or globules are known as bivalve serous cells, or pigment cells (Cheng, 1981, 1984). The bivalve serous cells are yellowish or brownish and may participate in the exclusion of lipids. These cells seem to be different from the morula-like cell in *T. crocea* or the type II cell in *T. maxima,* both of which are colorless. The morula-like cell and the type II cell have been observed only in the Tridacnidae, which suggests that this type of hemocyte may participate in the maintenance of zooxanthellae in giant clams.

The giant clams obtain most of the organic carbon required for growth from the photosynthetic fixation of carbon by zooxanthellae (Klumpp and Griffiths, 1994). The clam has to collaterally supply minerals and bicarbonates to the zooxanthellae for photosynthesis; thus these materials must be transported to the algae (Trench,

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* Current address: Marine Biotechnology Institute, Kamaishi Laboratories, 3-75-1 Heita, Kamaishi, Iwate 026, Japan.

1987). Cytochemical staining has shown that the large granules in the morula-like cell do not participate in the transport of sugars and lipids (Nakayama *et al.*, 1997). To understand the role of the morula-like cell in giant clam-zooxanthellae symbiosis, the proteins specifically contained in the morula-like cell, especially those in the large granules, were studied.

Materials and Methods

Animals

Tridacna derasa were purchased from the Palau Mariculture Demonstration Center (Republic of Palau). In the laboratory on the research vessel *Sohgen-maru*, hemocytes were immediately collected as described below.

Preparation and fractionation of hemocytes

Hemolymph was withdrawn from the pericardial chamber with a 50-ml syringe and an 18-gauge needle, and collected in 50-ml polypropylene centrifuge tubes containing EDTA solution (0.04% EDTA-2Na and 2.5% NaCl, pH 7.4) as an anti-coagulation reagent. Hemocytes were collected by centrifugation (700 × *g*, 5 min) and suspended in APSW, artificial Pacific seawater (Borowitzka and Larkum, 1976). The suspension of hemocytes was then layered on 50% Percoll (Pharmacia, Uppsala, Sweden) solution containing 2.5% NaCl. Morula-like cells were separated from eosinophilic granular hemocytes and agranular cells by centrifugation (700 × *g*, 15 min). The morula-like cells were obtained as a pellet, and the mixture of the eosinophilic granular hemocytes and agranular cells remained in the upper layer. After suspension in APSW, the morula-like cells were partially disrupted by sonication (40 W, 3 min) on ice. The resultant solution was layered on 50% Percoll solution and centrifuged (700 × *g*, 15 min). The pellet of Percoll density-gradient fractionation contained the debris of disrupted cells. The upper layer containing the large granules was collected, mixed with an equal volume of APSW, and recentrifuged to precipitate large granules.

Preparation of tissue extract

Tissues were dissected from the giant clam and extracted, using a Douce homogenizer, in Dulbecco's phosphate-buffered saline (Mg-, Ca-free). After removal of large debris by sedimentation, the extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Isolation of zooxanthellae

Zooxanthellae were isolated from the mantle of *T. derasa* by the method of Masuda *et al.* (1994). After disruption of the mantle tissue by means of a Polytron-type homogenizer (Ystral GmbH, Dottingen, Switzerland), the extract was filtered through 10-μm mesh. Zooxanthellae were contained in the filtrate and washed with APSW several times.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

After determination of protein concentration by a modified Lowry method (DC protein assay kit, Bio-Rad Labs., Hercules, California) according to the manufacturer's instructions, hemocyte and tissue samples were dissolved in SDS dissociation buffer (2.5% SDS, 10% glycerol, 5% 2-mercaptoethanol, 62.5 m*M* Tris-HCl, pH 6.8) to approximately equal protein concentrations. After heating at 95°C for 10 min, samples were electrophoresed by PhastSystem (Pharmacia, Uppsala, Sweden), according to the method of Laemmli (1970), on 20% SDS-polyacrylamide gels.

Amino acid sequencing

Proteins separated by SDS-PAGE were electrotransferred to polyvinylidene difluoride (PVDF) membrane and then visualized with 0.5% Coomassie brilliant blue R-250 solution. The appropriate stained bands were cut out, and the N-terminal amino acids sequence was determined by a gas-phase amino acid sequencer (PSQ-2, Shimadzu Co., Kyoto, Japan).

Polyclonal antibody

A peptide with 15 amino acids was designed from the determined N-terminal amino acid sequence of the 7.4-kDa protein and synthesized by the multiple antigenic peptide (MAP) method (Posnett *et al.*, 1988). The polyclonal antibody was raised by immunizing rabbits with the MAP.

Western blot analysis

The hemocytes or granule lysate was applied to SDS-PAGE, then electrotransferred to PVDF membrane. Immunoblotting was performed according to the procedure of Towbin *et al.* (1979). After being treated with the blocking solution (Block Ace, Snow Brand Milk Products, Hokkaido, Japan), blots were incubated overnight at 4°C with the anti-7.4-kDa protein rabbit polyclonal antibody at 1:100 dilution. Goat anti-rabbit IgG conjugated with horseradish peroxidase (Cappel, Organon Teknika Corp., Durham, North Carolina) at 1:200 dilution or an ABC-AP rabbit kit (Vector Labs., Burlingame, California) was used as a secondary antibody. The immunoreactive band was visualized with a peroxidase staining re-

agents kit (Konica co., Tokyo, Japan) or an alkaline phosphatase substrate kit IV (Vector Labs., California).

After being electrotransferred to PVDF membrane, the glycoprotein was oxidized with periodic acid and reacted with biotin-hydrazide by the method of Kondo *et al.* (1991), and the sugars were visualized by avidin conjugated with horseradish peroxidase using a commercial kit (G. P. Sensor, Honen co., Tokyo, Japan) according to the manufacturer's instructions.

Analysis of amino acid sequence

An amino acid sequence homology search was performed using the MPsearch program underlying the Smith-Waterman sequence comparison algorithm (Smith and Waterman, 1981) through e-mail servers at the National Cancer Center (Tokyo, Japan).

Results

Proteins specific to the morula-like cell

Three types of hemocytes (the eosinophilic granular hemocyte, the agranular cell, and the morula-like cell) were recognized in the hemolymph of *Tridacna derasa* (data not shown), as in *T. crocea*. After fractionation of the morula-like cell in *T. derasa* and subfractionation of its granules, each lysate was analyzed by SDS-PAGE. Comparison of the SDS-PAGE patterns of the morula-like cell and a mixture of the eosinophilic granular hemocyte and the agranular cell showed that proteins with molecular weights of 64 kDa, 17 kDa, and 7.4 kDa were specific to the morula-like cell (Fig. 1, lane 3). The 17-kDa protein was not found in the large-granule fraction of the morula-like cell but was in the cell-debris fraction (Fig. 1, lane 4). The 64-kDa and 7.4-kDa proteins were in both the cell-debris (Fig. 1, lane 4) and the large-granule fractions (Fig. 1, lane 5).

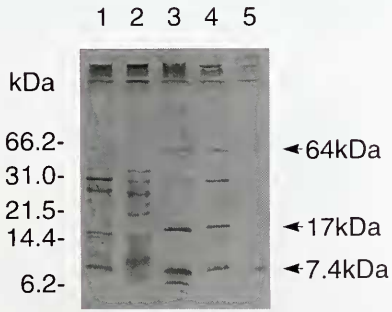

Figure 1. Identification of specific proteins in the morula-like cell. Hemocytes and fractionated samples were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions and stained with silver nitrate. Lane 1, hemocytes lysate; Lane 2, lysate of the eosinophilic granular hemocyte and the agranular cell; Lane 3, morula-like cell lysate; Lane 4, precipitate of Percoll density gradient centrifugation after disruption of the morula-like cell; Lane 5, lysate of large granules which were isolated from the morula-like cell. Arrows indicate 65-kDa, 17-kDa, and 7.4-kDa proteins specific to the morula-like cell. Each lane contained 0.34 μg protein except lane 5, which contained 0.1 μg protein.

N-Terminal amino acid sequence

After electrotransfer of proteins from SDS-polyacrylamide gels to PVDF membrane, the 17-kDa and 7.4-kDa bands were cut out and placed in the sequencer. Twenty-five amino acids and 30 amino acids were identified in the N-termini of the 17-kDa and the 7.4-kDa proteins, respectively. Both proteins were rich in tyrosine and phenylalanine. In the 7.4-kDa protein, aromatic amino acids appeared at almost every 10 amino acids (Fig. 2).

Immunodetection of the 7.4-kDa protein

Western blot analysis revealed that the polyclonal antibody raised against a synthetic peptide corresponding to the 8th to 22nd amino acids of the 7.4-kDa protein was immunoreactive against that protein (Fig. 3). Another protein having a molecular weight of about 16 kDa was also immunoreactive with the polyclonal antibody (Fig. 3, lane 4). This 16-kDa protein was not detected in the large-granule fraction but only in the cell-debris fraction of the morula-like cell. Because the 16-kDa and 17-kDa proteins migrated closely together on SDS-PAGE, the 16-kDa protein appeared only as a contaminant in the 17-kDa protein. Careful examination of the HPLC chart from the sequencer revealed that the sequence of the 16-kDa protein was Y-D-X-S-F-X-K-X-R-X-X-X-F-X-A-D-P-A-S-X-Q-N-Y-Y-R. This was identical to the sequence of the 7.4-kDa protein excepting the unidentified residues, but distinct from that of the 17-kDa protein. The lysate of eosinophilic granular hemocytes and agranular cells did not contain immunoreactive proteins (Fig. 3, lane 2)

Distribution of the 7.4-kDa protein

To analyze the tissue distribution of the 7.4-kDa protein, tissue extracts were analyzed by the immunoblotting technique. The hemocytes (Fig. 4, lane 1), the zooxanthellate portion of mantle (Fig. 4, lane 2), and the kidney (Fig. 4, lane 7) contained the immunoreactive protein. A heavily reactive band was also observed near the dye front of electrophoresis of the zooxanthellate portion of the mantle. No immunoreactive protein was detected in the azooxanthellate portion of mantle, gill, foot, and adductor muscle.

Characterization of immunoreactive protein in zooxanthellae

After zooxanthellae were isolated from mantle tissue, they were analyzed by immunoblotting and glycosylation

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                  1                   10                  20
17kd protein;     I-G-H-S-G-N-G-L-V-Y-Y-G-N-H-A-Y-L-F-S-A

                 21      25
                 -D-H-L-T-G

                  1                   10                  20
7.4kd protein;    Y-D-F-S-F-X-K-K-R-N-D-G-F-Y-A-D-P-A-S-H

                 21                  30
                 -Q-N-Y-Y-R-R-V-S-G-A
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Figure 2. Amino acid sequences of the 17-kDa and 7.4-kDa proteins in the morula-like cell. The one-letter code is used. X, unidentified.

detection techniques. Immunoreactive protein appeared as a broad band and a slightly smaller component (*ca.* 5 kDa) that migrated faster than 7.4 kDa (Fig. 5A, lane 2). The 16-kDa and 7.4-kDa proteins in the morula-like cells (Fig. 5B, lane 1) and the immunoreactive protein in the zooxanthellae (Fig. 5B, lane 2) were shown to be glycosylated.

Discussion

Molluscan hemocytes packed with large colorless granules have been observed only in giant clams (the Tridacnidae). This type of hemocyte is known as a type II cell in *Tridacna maxima* (Reade and Reade, 1976) and a morula-like cell in *T. crocea* (Nakayama *et al.*, 1997). Proteins with molecular weights of 64 kDa, 17 kDa and 7.4 kDa were specific to the morula-like cell of *T. derasa* and not associated with the eosinophilic granular hemocyte or the agranular cell. The 7.4-kDa protein is contained in the large granules. No homologous sequence was found in the homology search of the N-terminal amino acid sequences of these proteins (Fig. 2), which indicates that these are novel proteins. The polyclonal antibody that was immunoreactive against the 7.4-kDa protein in the large granule was also immunoreactive with the 16-kDa protein in the cytoplasm of the morula-like cell. The N-terminal sequence of this 16-kDa protein was identical to that of

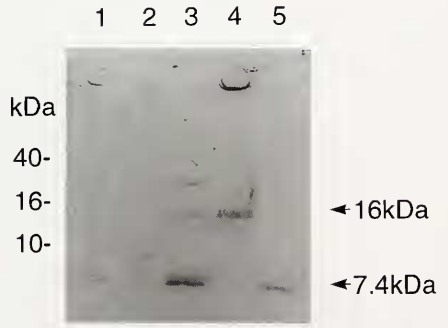

Figure 3. Immunoblot analysis of the 7.4-kDa protein specific to the morula-like cell. Hemocytes and the Percoll density-gradient fractions were electrophoresed, electrotransferred onto PVDF membrane, and immunologically stained. Lane 1, hemocytes lysate; Lane 2, lysate of eosinophilic granular hemocytes and agranular cells; Lane 3, morula-like cell lysate; Lane 4, precipitate of Percoll density gradient centrifugation of the morula-like cell lysate; Lane 5, the large granule lysate. Each lane contained 1.4 μg protein except lane 5, which contained 0.4 μg protein.

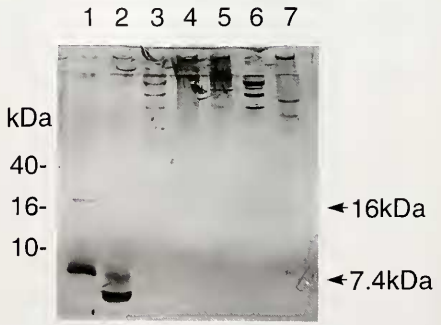

Figure 4. Immunoblot analysis of the 7.4-kDa protein in various tissues. Lane 1, morula-like cell; Lane 2, zooxanthellate portion of mantle; Lane 3, azooxanthellate portion of mantle; Lane 4, gill; Lane 5, foot; Lane 6, adductor muscle; Lane 7, kidney. Note that the zooxanthellate portion of mantle contained a large amount of immunoreactive proteins. Each lane contained 0.34 μg protein.

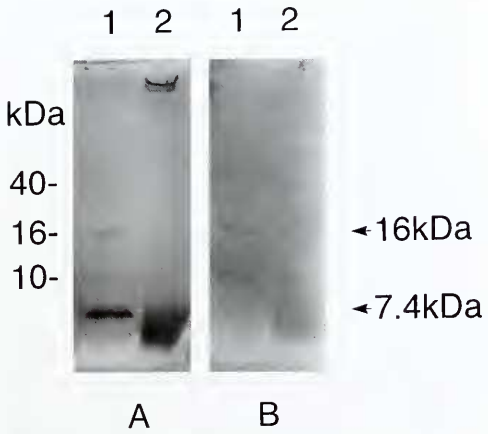

Figure 5. Immunoblot analysis and carbohydrate staining of the 7.4-kDa protein in the morula-like cell and zooxanthellae. (A) Immunological staining; (B) Detection of glycosylated proteins. Lane 1, morula-like cell; Lane 2, zooxanthellae. Each lane contained 1.4 μg protein.

7.4-kDa protein but distinct from that of the 17-kDa protein. This suggests that this protein may be synthesized as 16 kDa. After transport into granules, the C-terminal region of the 16-kDa protein may undergo proteolytic maturation to 7.4-kDa. Detection of sugars by the biotin-hydrazide method indicates that 16-kDa and 7.4-kDa proteins are glycosylated.

The morula-like cells have been observed in several species of symbiotic clam in the families Tridacnidae and Cardiidae: *Tridacna maxima* (Reade and Reade, 1976); *T. crocea* (Nakayama *et al.*, 1997); *T. derasa, Hippopus hippopus, H. porcellunas, Corculum cardissa,* and *Fragum fragum* (our unpublished observations). The morula-like cell is always observed in the hemolymph of *T. crocea* and constitutes 6% of all the hemocytes (Nakayama *et al.*, 1997). This observation suggests that the morula-like cell may play a role in the giant clam-zooxanthellae symbiosis. Immunodetection showed that the amount of protein immunoreactive to the anti-7.4-kDa antibody and of a slightly lower molecular weight (*ca.* 5 kDa) was far greater in the surface layer of mantle containing zooxanthellae than in mantle tissue without zooxanthellae. The immunoreactive proteins were also detected in zooxanthellae and kidney, although their molecular weights were lower than 7.4 kDa. Some zooxanthellae grown in the zooxanthellal tube may be digested in the stomach or in the siphonal mantle (Maruyama and Heslinga, 1997). Other studies report finding zooxanthellae in the blood vessels of the kidney (Trench *et al.*, 1981) or in amoebocytes within the kidney (Morton, 1978). Although these observations indicate that the 7.4-kDa protein may be in a digestive mass, the absence of phagocytic ability in the morula-like cell (Nakayama *et al.*, 1997) is evidence that the 7.4-kDa protein in these cells is not derived from phagocytic digestion of zooxanthellae. Nevertheless, the possibility that the morula-like cell takes up proteins secreted from eosinophilic granular hemocytes that digest zooxanthellae cannot be ruled out.

The 7.4-kDa protein was rich in aromatic amino acids in the N-terminal region and was characterized by alignment of aromatic amino acids at almost every 10th residue. Tyrosine-rich proteins have been reported in some invertebrates. The serous cell of *Crassostrea virginica* contains tyrosine-rich protein in the granules, which are composed of a complex of lipids and proteins with properties similar to those of lipofuscins (Cheng, 1981, 1984). The morula-like cell, although morphologically similar to the serous cell, lacks lipid in the large granules, as indicated by Sudan black B staining (Nakayama *et al.*, 1997). Thus the morula-like cell is not functionally similar to the serous cell. Tyrosine-rich proteins are also found in *Geukensia demissa* (Waite, 1977). The proteins, which are located in the shell cuticle, are suggested to be hardened by quinone tanning. When the hemolymph of *T. derasa* or *T. crocea* was mixed with seawater *in vitro,* the hemocytes aggregated to make large clots. In the aggregation process, some of the morula-like cells released the large granules, which burst and discharged their contents (Nakayama *et al.*, 1997). Although it is possible that the tyrosine residues in the 7.4-kDa protein are substrate for protein crosslinking, phenol oxidase activities have not been observed in the hemocytes and hemolymph of *T. derasa* or *T. crocea* (unpubl. data). The molecular weight of immunoreactive protein in the morula-like cell was larger than in the mantle. The 7.4-kDa protein may be synthesized in the morula-like cell, secreted in the mantle, bound to the zooxanthellae in the zooxanthellal tube, and finally, transported to the kidney with the zooxanthellae. Alternatively, the 7.4-kDa protein may be synthesized in the zooxanthellae and taken into the morula-like cell where it is modified by a process such as glycosylation, increasing its molecular weight. To clarify the function of the proteins specific to the morula-like cell, the complete amino acid sequences and the location of the genes coding these proteins must be determined.

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